FEBS 18024 FEBS Letters 400 (1997) 355–358

# Fructan synthesis in transgenic tobacco and chicory plants expressing barley sucrose:fructan 6-fructosyltransferase

Norbert Sprenger[a], Luisa Schellenbaum[a], Kees van Dun[b], Thomas Boller[a], Andres Wiemken[a,*]

[a]*Department of Botany, University of Basel, Hebelstrasse 1, CH-4056 Basel, Switzerland*
[b]*Department of Biotechnology, D.J. van der Have B.V., 4410 AA Rilland, The Netherlands*

Received 28 October 1996

**Abstract We have recently cloned a cDNA encoding sucrose:-fructan 6-fructosyltransferase (6-SFT), a key enzyme of fructan synthesis forming the β-2,6 linkages typical of the grass fructans, graminans and phleins [Sprenger et al. (1995) Proc. Natl. Acad. Sci. USA 92, 11652–11656]. Here we report functional expression of 6-SFT from barley in transgenic tobacco and chicory. Transformants of tobacco, a plant naturally unable to form fructans, synthesized the trisaccharide kestose and a series of unbranched fructans of the phlein type (β-2,6 linkages). Transformants of chicory, a plant naturally producing only unbranched fructans of the inulin type (β-2,1 linkages), synthesized in addition branched fructans of the graminan type, particularly the tetrasaccharide bifurcose which is also a main fructan in barley leaves.**

*Key words:* Fructan; Sucrose:fructan 6-fructosyltransferase; *Nicotiana tabacum*; *Cichorium intybus*; *Hordeum vulgare*

## 1. Introduction

Fructans are a common, structurally diverse class of oligo- and polysaccharides based on fructose [1,2]. In the plant kingdom, only a limited number of species are able to form fructans, among them many members of the evolutionarily advanced and prominent orders of Asterales, Liliales and Poales [3]. In plants capable of forming them, fructans play an important role as carbohydrate reserves alternative or in addition to starch, and they may also fulfill more subtle roles in the regulation of short-term carbohydrate partitioning and osmoregulation; furthermore, they are suspected to contribute to tolerance against drought and freezing stress [3–5].

Our current understanding of fructan biosynthesis in plants is that they essentially represent extensions of sucrose by sucrose-derived fructosyl units [5]. The inulin-type fructans of the Asterales are extensions of sucrose through β-2,1-linked fructosyl units; the characteristic inulin series of different DP is generated by the consecutive action of sucrose:sucrose 1-fructosyltransferase (1-SST) which catalyzes the essentially irreversible formation of isokestose, and fructan:fructan 1-fructosyltransferase (1-FFT) which catalyzes reversible transfructosylations between fructans of different chain lengths [6–8]. The inulin neoseries of the Liliales is produced similarly by fructan:fructan 6-glucose fructosyltransferase (6-G-FFT) which transfers a fructosyl unit from a fructan to the 6- position of the glucosyl moiety of sucrose, forming neokestose [9]. The graminan- and phlein-type fructans represent extensions of sucrose through β-2,6-linked fructosyl units, with or without branches of β-2,1-linked fructosyl units, and appear to be synthesized by the consecutive, irreversible actions of 1-SST and a newly identified sucrose:fructan 6-fructosyltransferase (6-SFT) [10–12], with or without subsequent action of fructan exohydrolases which may trim the β-2,1 branches [5].

Recently, we have cloned a cDNA encoding 6-SFT from barley [12]. Transient expression of this cDNA in *Nicotiana* protoplasts yielded fully functional 6-SFT, as shown by enzymatic assays in extracts [12]; however, these protoplasts did not accumulate any fructan. Here we report that functional expression of barley 6-SFT in transgenic plants leads to fructan accumulation in tobacco and alters the pattern of fructans in chicory.

## 2. Material and methods

### 2.1. Transformation and plant material

Barley sucrose:fructan 6-fructosyltransferase cDNA [12] was subcloned into a plant binary plasmid vector, containing a NOS NPTII gene as selectable marker, between a 35S CaMV promoter and a *nos* terminator sequence. This vector construct was used to transform leaf discs of *Nicotiana tabacum* var. Samson NN and *Cichorium intybus* by *Agrobacterium tumefaciens* strain LB4044 following published procedures [13,14]. Chicory transformation was carried out at the biotechnology department of Nunhems Zaden, Haelen, The Netherlands. Regenerating tobacco and chicory shoots were selected for kanamycin resistance on MS medium [15], and chicory was transferred onto MS medium without kanamycin to induce root formation. Primary transformants of tobacco and chicory were transferred into commercial organic soil, grown in a greenhouse, and used for fructan analysis (chicory) and seed production (tobacco). Young leaves from primary chicory transformants were induced to accumulate fructans by putting excised leaves into 100 mM sucrose in continuous light for up to 48 h [16]. Seeds from selfed transgenic tobacco plants (S1 generation) were surface sterilized by submersion in 70% ethanol for 2 min and 13% NaOCl for another 8 min followed by washing 5 times in sterile water. Thereafter they were germinated on MS medium containing 100 mg $l^{-1}$ kanamycin. After 4 weeks, growing plants (representing homozygous or heterozygous transformants) were transferred to a substrate consisting of 80% coarse sand, 10% quartz sand and 10% clay. The plants were kept in a growth chamber (16 h light period; PAR 220 μmol $m^{-2}$ $s^{-1}$; 25°C/18°C; 65% relative humidity) for 60 days and fertilized weekly with a Hoagland nutrient solution containing 10% phosphorus prior to analysis of fructans.

### 2.2. Extraction of soluble carbohydrates

Plant material was transferred into liquid nitrogen immediately after harvesting and either kept at −80°C or lyophilized (S1 generation of tobacco) prior to analysis. For extraction of soluble carbohydrates either frozen plant material ground in liquid nitrogen (200–300

*Corresponding author. Fax: (41) (61) 267 23 30.
E-mail: wiemken@ubaclu.unibas.ch

*Abbreviations:* 1-FFT, fructan:fructan 1-fructosyl transferase; 6-SFT, sucrose:fructan 6-fructosyltransferase; 1-SST, sucrose:sucrose 1-fructosyltransferase; DP, degree of polymerization; DW, dry weight


*PII* S0014-5793(96)01418-4

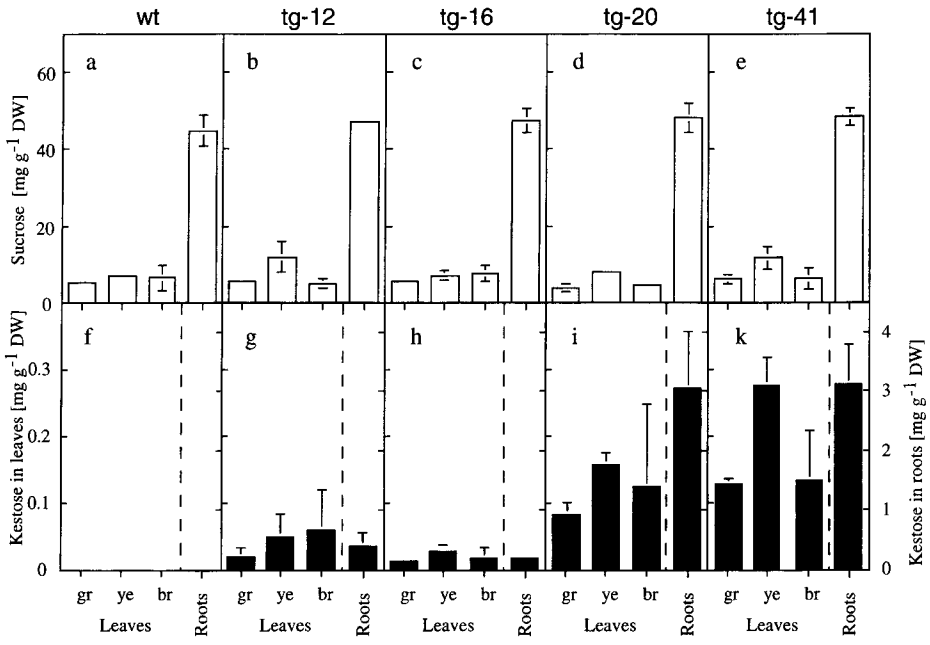


Fig. 1. Sucrose (a–e) and kestose (f–k) content in leaves and roots of untransformed tobacco (wt) and four independent tobacco lines expressing barley 6-SFT (tg-12, tg-16, tg-20, tg-21). Green (gr), yellow (ye) and brown (br) leaves were analyzed separately. Data represent mean values ± S.E. for four plants. Note different scale for kestose in leaves and roots.

mg fresh weight) or lyophilized and ground material (20–30 mg dry weight) was extracted twice with 80% (v/v) methanol and twice with 20% (v/v) methanol in portions of 0.5 ml at 65°C for 15 min each time. The pooled extracts were lyophilized and the extracts were redissolved in water (0.25 ml or 1 ml per g fresh weight or dry weight respectively). To adsorb phenolic substances, insoluble polyvinylpyrrolidone (PVP-AT, Serva, Heidelberg, Germany) was included in all the steps. After treating the extracts with an anion and cation exchange resin (Serdolit MB, Serva, Germany) the resulting supernatant was further extracted with water-saturated *n*-butanol and the final water phase was analyzed by HPLC.

### 2.3. *Analysis of soluble carbohydrates*

Analysis of carbohydrates by HPLC was done on an anion exchange column PA-1 (Dionex, Sunnyvale, CA, USA) using a Dionex HPLC system DX300 equipped with an amperometric detector. Monosaccharides were eluted in a gradient of NaOH from 100 mM to 300 mM in 5 min at a flow rate of 1 ml $min^{-1}$. Subsequently disaccharides and carbohydrates with a DP > 2 were separated by a gradient of 0–500 mM sodium acetate in 300 mM NaOH from 5 to 30 min, at the same flow rate. Figs. 2 and 3 show the region of the chromatograms between 10 and 30 min, in which fructans are eluted. Product identification and quantification were done using retention times and response factors that were determined with pure and defined standards [11] and with trehalose as an internal or external standard. Furthermore some extracts were co-chromatographed with defined oligofructosyl sucroses (isokestose[1], kestose, bifurcose and 6b-kestin) and with soluble carbohydrate extracts from *Helianthus tuberosus* tubers and *Dactylis glomerata* stems. A soluble carbohydrate extract from barley growth zones was used for comparison.

[1] We follow the nomenclature proposed by Lewis [1]: isokestose for the trisaccharide *O*-β-D-fructofuranosyl-(2→1)-β-D-fructofuranosyl-(2′1)-α-D-glucopyranoside; kestose for *O*-β-D-fructofuranosyl-(2→6)-β-D-fructofuranosyl-(2′1)-α-D-glucopyranoside; bifurcose or 6a-isokestin for *O*-β-D-fructofuranosyl-(2→1)-*O*-(β-D-fructofuranosyl-(2→6))-β-D-fructofuranosyl-(2′1)-α-D-glucopyranoside; 6b-kestin for *O*-β-D-fructofuranosyl-(2→6)-β-D-fructofuranosyl-(2→6)-β-D-fructofuranosyl-(2′1)-α-D-glucopyranoside.

## 3. Results and discussion

### 3.1. *The fructan trisaccharide kestose is formed in transgenic tobacco*

Among the kanamycin-resistant primary transformants of tobacco which contained the barley 6-SFT construct, nine were found that exhibited 6-SFT activity (5–25 pkat $mg^{-1}$ protein) and contained the fructan trisaccharide kestose in leaf extracts (data not shown). Two independent transformants with high kestose levels and two with low kestose levels were selected and selfed. The kanamycin-resistant progeny (S1 generation) of these transformants was indistinguishable from untransformed wild-type plants. To examine fructan synthesis, these S1 plants were harvested at the stage of flower induction, and the composition of soluble carbohydrates was analyzed separately in roots and green, yellow and brown leaves. Sucrose was the predominant sugar in all samples; it amounted to about 5–10 mg $g^{-1}$ dry weight in the leaves and to about 50 mg $g^{-1}$ dry weight in the roots, and there was no difference between the transformants and the untransformed wild-type (Fig. 1a–e). In contrast, the fructan trisaccharide kestose was present only in samples from the transformants (Fig. 1f–k).

Consistently, progeny of the primary transformants with low kestose levels (tg-12 and tg-16) had low levels of kestose in all types of leaves and in roots, while progeny of the high-kestose transformants (tg-20 and tg-21) had comparatively high levels of kestose. Interestingly, for all transformants, the kestose level in roots was about an order of magnitude higher than the kestose level in leaves. This may be connected to the high sucrose content of roots compared to that of the leaves; it is well-known that 6-SFT depends on high concentrations of sucrose for efficient kestose production [10,11]. It is also worth noting that for each transformant, kestose levels were higher in yellow senescent leaves than in green ones, and

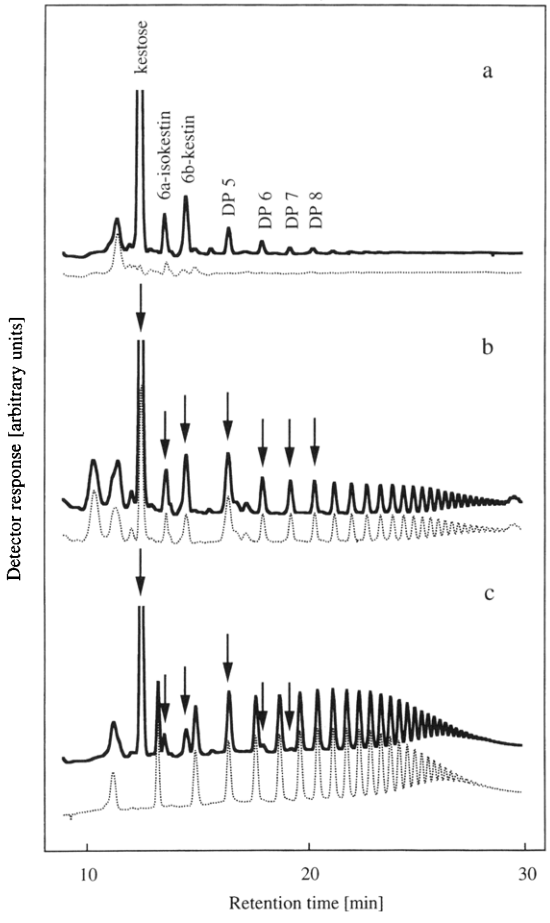


Fig. 2. HPLC analysis of fructans in roots from a tobacco line expressing 6-SFT. (a) Root extract from the transformed line tg-20 (bold line) yields a series of peaks at retention times of known fructans (named above the peaks) which are absent, or almost absent, in a corresponding root extract from untransformed tobacco (dotted line). (b, c) Co-chromatography of the fructans formed in transgenic tobacco with phlein and inulin. Dotted lines represent an extract from *Dactylis glomerata* containing phlein-type fructans (b) and an extract from *Helianthus tuberosus* containing inulin-type fructans (c); bold lines represent mixtures of these fructans and the root extract from the transgenic tobacco shown in (a). Arrows indicate new peaks in the transgenic tobacco, named in (a).

still substantial although quite variable in brown desiccated leaves (Fig. 1f–k) while very young leaves contained almost no kestose (data not shown). This indicates that tobacco leaves lack enzymes for kestose degradation, such that the compound accumulates over the whole lifespan of the leaves; it may also explain why *Nicotiana* mesophyll protoplasts expressing barley 6-SFT transiently for 24 h did not produce measurable levels of kestose [12].

### 3.2. *Roots of transgenic tobacco synthesize phlein-type fructans*

Preliminary experiments had shown that root extracts from transgenic tobacco, which contained high levels of kestose, also contained additional fructans. This was analyzed more closely in root extracts from S1 generation plants of the high-kestose transformant tg-20 (Fig. 2). Although kestose was the predominant oligosaccharide in these extracts, the chromatogram revealed the presence of a number of larger oligosaccharides that were absent in corresponding root extracts from untransformed tobacco (Fig. 2a). The first two of them were identified by co-chromatography with pure and defined standards as bifurcose (6a-isokestin) and 6b-kestin; the larger ones were tentatively assigned to a series of fructan oligosaccharides of the phlein type (β-2,6 linkages) with DP 5–8. To test this further, the extracts were mixed with an extract from orchard grass (*Dactylis glomerata*) containing β-2,6-linked phlein-type fructans, or with an extract from Jerusalem artichoke tubers (*Helianthus tuberosus*) containing β-2,1-linked inulin-type fructans. Clearly, the peaks in the root extract from the transgenic tobacco perfectly matched the phlein series (Fig. 2b) but not the inulin series (Fig. 2c).

Surprisingly, a small amount of the branched fructan trisaccharide bifurcose (6a-isokestin) appeared to be present in the transgenic tobacco roots (Fig. 2a,b). Possibly a trace of isokestose, which is known to be formed in small amounts by side reactions of invertases [17], was trapped by 6-SFT as fructosyl acceptor substrate resulting in the production of

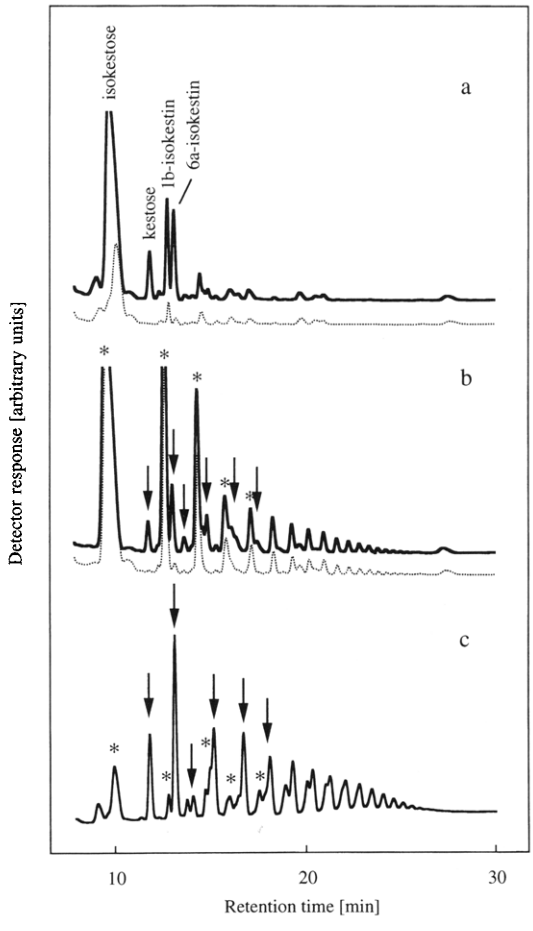


Fig. 3. HPLC analysis of extracts from excised leaves of chicory expressing 6-SFT, induced to accumulate fructans by continuous illumination in the presence of 100 mM sucrose. (a) After 24 h of illumination, the transformed line 1102 (bold line) yields four peaks at retention times of known fructans (named above the peaks), only two of which, isokestose and 1b-isokestin, are also present in the corresponding untransformed line (dotted line). (b) After 48 h of illumination, the transformed line 1102 (bold line) contains the fructans of the inulin series similarly present in the corresponding untransformed line (dotted line), but in addition also a series of branched fructans. (c) Chromatogram of fructans extracted from barley leaf growth zones naturally expressing 6-SFT, shown for comparison. In (b,c), stars indicate fructans of the inulin series, arrows indicate kestose and branched graminans naturally produced in barley leaves.

bifurcose. In barley leaves, isokestose is produced by sucrose:sucrose 1-fructosyltransferase (1-SST), and this compound appears to be the preferred fructosyl-acceptor substrate for 6-SFT [10–12].

### 3.3. *Chicory transgenic for 6-SFT forms mixed-type fructans in addition to its natural inulin-type fructans*

Knowing that barley 6-SFT synthesizes fructans most efficiently in the presence of isokestose or other fructans as fructosyl acceptors and that it acts primarily as β-fructosidase if sucrose is present as a sole substrate [11,12], we were interested to examine the effect of expressing barley 6-SFT in a fructan-producing plant. Several transformants were obtained from chicory, a plant producing β-2,1-linked inulin. The effect of the transgene was studied in excised leaves, induced to accumulate fructans by continuous illumination in the presence of 0.1 M sucrose (Fig. 3). After 24 h of illumination, leaves of control plants started to accumulate the first members of the inulin series, isokestose, 1b-isokestin; the transgenic plants had two peaks in addition that were absent or almost absent from control plants, namely kestose and the branched fructan tetrasaccharide bifurcose (6a-isokestin) (Fig. 3a). Bifurcose is the main product formed by purified barley 6-SFT in the presence of sucrose and isokestose [10–12]. After 48 h of illumination, the control leaves accumulated fructans of the inulin series; the transgenic leaves expressing 6-SFT now contained, in addition, not only kestose and bifurcose but also additional peaks corresponding to branched fructans of higher DP, most likely resulting from 6-fructosylation of the inulins generated by the chicory enzymes (Fig. 3b). For comparison, Fig. 3c shows a chromatogram of soluble oligosaccharides in barley leaf growth zones, which are composed of inulin-type fructans as well as branched graminan-type fructans [18]. Kestose and bifurcose did not increase further in leaves from the transgenic plants between 24 and 48 h. This may be due to competition of different fructosyl transferases for the substrate, sucrose. In particular, the 1-FFT activity induced after 24 h, which is responsible for the accumulation of inulin-type fructans and has a high affinity for sucrose as acceptor substrate [6,19], may out-compete the heterologous 6-SFT. This type of competition may also explain the fact that the storage roots of the transformed chicory plants contained large quantities of inulins but no detectable products of 6-SFT (data not shown).

The fact that bifurcose and higher branched-chain fructans accumulate in the transformed chicory leaves (Fig. 3a,b) provides an indication that the heterologously expressed barley 6-SFT is located in the same cell compartment as the endogenous 1-SST and 1-FFT which are normally responsible for the formation of isokestose and inulin-type fructans of a higher DP in Asterales [7,8]. 1-SST has been shown to be a vacuolar enzyme in barley [20] as well as in tubers of Jerusalem artichoke [21], a close relative of chicory. The heterologous 6-SFT therefore appears to be targeted to the vacuole in chicory, and most likely also in the transformed tobacco. Vacuolar targeting of 6-SFT may explain the lack of a visible phenotype of the transformants. Tobacco and potato plants producing the fructan levan upon heterologous expression of bacterial levan sucrase, without special targeting sequences, showed phenotypic changes such as necrosis or stunted growth, most probably because fructans accumulated in cell compartments more vulnerable to interference with general metabolism than the vacuoles [22,23].

*Acknowledgements:* We are most grateful to Erika Roth for typing the manuscript. This work was supported by Swiss National Science Foundation Grant 31-27923.89.

## References

[1] Lewis, D.H. (1993) New Phytol. 124, 583–594.
[2] Suzuki, M. and Chatterton, N.J. (1993) Science and Technology of Fructans, CRC Press, Boca Raton, FL.
[3] Hendry, G.A.F. (1993) New Phytol. 123, 3–14.
[4] Housley, T.L. and Pollock, C.J. (1993) in: Science and Technology of Fructans (Suzuki, M. and Chatterton, N.J., Eds.) pp. 191–225, CRC Press, Boca Raton, FL.
[5] Wiemken, A., Sprenger, N. and Boller, T. (1995) in: International Symposium on Sucrose Metabolism (Pontis, H.G., Salerno, G.L. and Echeverria, E.J., Eds.) pp. 179–188, American Society of Plant Physiology Press, Rockville, MA.
[6] Lüscher, M., Frehner, M. and Nösberger, J. (1993) New Phytol. 123, 717–724.
[7] Koops, A.J. and Jonker, H.H. (1996) Plant Physiol. 110, 1167–1175.
[8] Lüscher, M., Erdin, C., Sprenger, N., Hochstrasser, U., Boller, T. and Wiemken, A. (1996) FEBS Lett. 385, 39–42.
[9] Vijn, I., van Dijken, A., Sprenger, N., van Dun, K., Weisbeek, P., Wiemken, A. and Smeekens, S. (1996) Plant J. (in press).
[10] Simmen, U., Obenland, D., Boller, T. and Wiemken, A. (1993) Plant Physiol. 101, 459–468.
[11] Duchateau, N., Bortlik, K., Simmen, U., Wiemken, A. and Bancal, P. (1995) Plant Physiol. 108, 1249–1255.
[12] Sprenger, N., Bortlik, K., Brandt, A., Boller, T. and Wiemken, A. (1995) Proc. Natl. Acad. Sci. USA 92, 11652–11656.
[13] Horsch, R.B., Fry, J.E., Hoffmann, N.L., Eichholtz, D., Rogers, S.G. and Fraley, R.T. (1985) Science 227, 1229–1231.
[14] Vermeulen, A., Vaucheret, H., Pautot, V. and Chupeau, Y. (1992) Plant Cell Rep. 11, 243–247.
[15] Murashige, T. and Skoog, F. (1962) Physiol. Plant. 15, 473–497.
[16] Wagner, W., Wiemken, A. and Matile, P. (1986) Plant Physiol. 81, 444–447.
[17] Obenland, D.M., Simmen, U., Boller, T. and Wiemken, A. (1993) Plant Physiol. 101, 1331–1339.
[18] Roth, A., Lüscher, M., Boller, T. and Wiemken, A. (1997) New Phytol. (in press).
[19] Koops, A.J. and Jonker, H.H. (1994) J. Exp. Bot. 45, 1623–1631.
[20] Wagner, W., Keller, F. and Wiemken, A. (1983) Z. Pflanzenphysiol. 112, 359–372.
[21] Frehner, M., Keller, F. and Wiemken, A. (1984) J. Plant Physiol. 116, 197–208.
[22] Ebskamp, M.J.M., van der Meer, I.M., Spronk, B.A., Weisbeek, P.J. and Smeekens, S.C.M. (1994) Bio/Technology 12, 272–275.
[23] van der Meer, I.M., Ebskamp, M.J.M., Visser, R.G.F., Weisbeek, P.J. and Smeekens, S.C.M. (1994) Plant Cell 6, 561–570.